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**ENHANCEMENT OF CELL MEDIATED
IMMUNITY THROUGH NON-SPECIFIC
IMMUNOSTIMULATION WITH
LIPOSOME ENCAPSULATED
GAMMA-INTERFERON (U)**

BY

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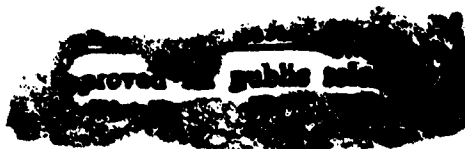
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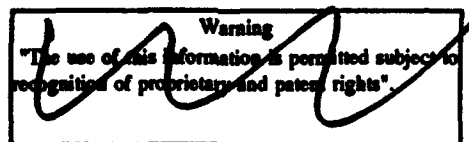
**Enhancement of Cell Mediated Immunity through Non-Specific
Immunostimulation with Liposome Encapsulated
Gamma-Interferon.**

by

**J.P. Wong, B. Kournikakis, E.G. Saravolac[†],
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ABSTRACT

The ability of liposome-encapsulated gamma interferon (LIP- γ IFN) to stimulate mouse cell-mediated immunity was assessed both *in vivo* and *in vitro*. The enhancement of the cell-mediated immune response was demonstrated *in vitro* by a chemiluminescent assay which measured the phagocytic activity of peritoneal macrophages. Peritoneal macrophages harvested from mice treated with gamma interferon (γ IFN) or muramyl dipeptide showed significant increases in both macrophage yield as well as in ability to phagocytize zymosan particles. However, when treated with γ IFN encapsulated within liposomes both macrophage yield and phagocytic activity further increased by at least 100% over unencapsulated γ IFN. Using the *in vivo* influenza mouse protection model, intranasally administered LIP- γ IFN resulted in a 70% survival rate to mice challenged intranasally with 10 LD₅₀ doses of influenza A/PR/8 virus compared with a 20% survival rate with free γ IFN. Together these results suggest that liposome encapsulation increases γ IFN efficacy in providing non-specific stimulation of the cell-mediated immune system.

RÉSUMÉ

La capacité de l'interféron gamma encapsulé dans des liposomes (LIP- γ IFN) de stimuler l'immunité à médiation cellulaire chez la souris a été évaluée *in vivo* et *in vitro*. L'accroissement de la réponse anticorps à médiation cellulaire a été mis en évidence *in vitro* par un test de chimioluminescence permettant de mesurer l'activité phagocytaire des macrophages péritonéaux. Les macrophages péritonéaux récoltés chez les souris traitées avec l'interféron gamma (γ IFN) ou le muramyl dipeptide ont présenté une augmentation substantielle tant du rendement en macrophage que de leur capacité de phagocyter des particules de zymosan. Toutefois, le traitement par γ IFN encapsulé dans des liposomes a fait augmenter ces deux paramètres d'au moins 100 p. 100 par rapport aux valeurs obtenues avec l' γ IFN non encapsulé. Dans le modèle murin de protection contre la grippe *in vivo*, le LIP- γ IFN administré par voie intranasale a fait augmenter à 70 p. 100 le taux de survie des souris exposées par voie intranasale à des doses de l'ordre de 10 LD₅₀ du virus grippal A/PR/8, comparativement à un taux de survie de 20 p. 100 avec l' γ IFN seul. Ces résultats indiquent que l'encapsulation dans des liposomes augmente l'efficacité de l' γ IFN en ce qui a trait à la stimulation non spécifique du système immunitaire à médiation cellulaire.

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INTRODUCTION

Immunotherapy has played an increasingly important role in clinical applications in recent years particularly where the immune therapy of AIDS and cancer are concerned [2]. However it appears that this approach has not been fully exploited for the prevention and treatment of viral, bacterial and infectious diseases. Immunotherapy may be categorized in two broad classes, specific and non-specific. Specific immunotherapy provides specific immunity to particular antigens using active immunization or adoptive transfer of sensitized lymphoid cells of specific immunoglobulins from immunized donors. In non-specific immunotherapy, the immune system of the host is normally enhanced by the administration of immunomodulators and thus has the potential to provide a general prophylaxis against a wide spectrum of infectious microorganisms. The ability to enhance immune competence is particularly significant in the defence against biological warfare (BW) agents as it may be possible to defend the host against a range of infectious entities; viral, bacterial or parasitic.

Immunomodulators such as gamma interferon (γ IFN), macrophage activating factor or muramyl dipeptide (MDP) operate by activating macrophages and as such yield an immunomodulated state reflected by the increase in the phagocytic function of these cells [6,7]. Macrophages are the body's primary line of natural defence against microbial infections. In addition to their ability to phagocytise microorganisms, macrophages play an essential role in humoral immunity by processing and presenting antigens to T-lymphocytes [3]. The use of immunomodulators to stimulate macrophage activity *in vivo* is confounded by a number of factors. Certain immunomodulators such as γ IFN do not enter target tissues efficiently [18] or are rapidly cleared from the general circulation and body following systemic administration [13,16].

In order to circumvent these effects immunomodulators may be incorporated into liposomes. Liposomes are microscopic lipid vesicles which have a number of characteristics making them an attractive carrier/delivery system for immunotherapeutic agents [4,16]. Immunomodulators may be encapsulated within liposomes and thus be protected from *in vivo* degradation and dilution [13,14]. Agents are readily released from liposomes in a gradual and sustained manner thus increasing their prophylactic/therapeutic

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efficiency while reducing their intrinsic toxicities [13,20]. In addition, liposomes are particularly well suited as carriers of macrophage stimulating agents as they are preferentially taken up by the macrophages upon systemic, pulmonary or peritoneal administration and thus the immunomodulators can be delivered to these macrophages in concentrated levels [4,16].

This study evaluates the effectiveness of liposomes in potentiating the immunomodulation effect of γ IFN. The stimulation of macrophage phagocytic activity by these immunomodulators in the mouse peritoneum is first assessed. Following that, the ability of pulmonary delivered liposome encapsulated and non-encapsulated γ IFN's are compared for prophylactic efficacy in the resistance of the mouse against an influenza virus.

MATERIALS AND METHODS

Materials. Gamma interferon was purchased from Boehringer Mannheim Canada (Dorval, PQ). Muramyl dipeptide and all phospholipids and cholesterol used for the study were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RPMI 1640 with glutamine was purchased from Flow Laboratories Inc. (Mississauga, Ont.) and was prepared by adding 2.0 g of sodium bicarbonate per litre of RPMI 1640. Lavage fluid for peritoneal washes (100 mL) contained 90 mL RPMI 1640, 10 mL fetal bovine serum (FBS) (Flow Laboratories, Mississauga, Ont.) and 0.0738 g sodium chloride. Harvested cells were washed in RPMI 1640 with 10% FBS. These solutions were filter sterilized with a 0.45 μ m Nalgene filter (Nalge Company, Rochester, NY.) and stored at 4°C.

Zymosan was prepared by suspending 150 g of zymosan (Sigma Chemical Co.) in 30 mL of phosphate buffered saline (PBS), pH 6.75 (Oxoid Canada, Ottawa, Ont.) and the suspension was boiled for 30 min. PBS was then added to restore the total volume to 30 mL. Ten mL aliquots were pelleted by centrifugation at 300 x g for 10-15 min in a Beckman TJ-6 centrifuge. Activated zymosan was prepared by incubating the zymosan

pellet at 37°C for 30 min with 2-3 mL normal human serum obtained from healthy adult volunteers. After incubation with the serum, the suspension was re-centrifuged as described above. The activated zymosan pellets were resuspended in 50 mL of RPMI to yield a working concentration of 1 mg/mL.

Animals. Six week old female Balb/c mice were purchased from Charles River Canada (St. Constant, PQ). Mice were acclimated for one week in the DRES vivarium before use and used over a four week period. Care and handling of mice followed guidelines set out by the Canadian Council on Animal Care.

Liposome Preparation. Liposomes used in this study for the encapsulation of γ IFN were prepared using a modification of the freeze-drying method described by Kirby and Gregoriadis [8]. Negatively charged liposomes were prepared using phosphatidylcholine: cholesterol: phosphatidylserine in a molar ratio of 7:2:1. Briefly, a total of 20 μ moles of the lipids in chloroform:methanol (2:1, v/v) were dried by heating at 45°C. Throughout this procedure, the contents of the tube was purged whenever possible with a gentle stream of dry nitrogen. The lipids were further dried for 30 min in a vacuum oven to remove residual organic solvent. The lipids were then rehydrated with 1 mL of distilled water. The γ IFN to be encapsulated (total volume of 400 μ L, 100,000 U/mL) was then added to the lipid mixture and freeze-dried overnight. The mixture was reconstituted in 100 μ L of 100 mM HEPES buffer in normal saline, pH 6.7 and vortexed for 2-3 min. After leaving the reconstituted liposomes at room temperature for 1 hr, they were washed with 8 mL of HEPES buffer and ultracentrifuged at 125,000 x g for 30 min at 4°C in a Beckman L8-70 ultracentrifuge fitted with a 70.1 Ti rotor. HEPES buffer was used to wash the pellet and then the liposomes were ultracentrifuged again as described above. The liposome pellet was reconstituted in 1 mL of HEPES buffer. The liposomes were negatively stained with 2% sodium phosphotungstate (pH 7.4) and the morphology and vesicle size distribution were analyzed by electron microscopy. Liposomes prepared using this method were found to be heterogeneous in size with the vesicle diameters ranging from approximately 300 nm to 2 μ m.

Macrophage Stimulation. 0.5 mL of 1 mg/mL MDP in PBS was administered by intraperitoneal (i.p.) injection to each mouse. Nine mice were injected for each test group to obtain 3 treated mice for each of the 24, 48 and 72 hr time points. The control group consisted of an equal number of mice injected with 0.5 mL PBS.

Macrophage Isolation. Peritoneal macrophages were prepared by a modification of the procedure described by Mishell and Shiigi [11]. The recovered lavage fluid for each mouse was pooled. The lavage fluid was then centrifuged at 160 x g for 7 min. The resulting cell pellet was resuspended in RPMI 1640 plus 10% FBS and centrifuged again as described above. The washed pellet was resuspended in RPMI 1640 to a final volume of 3 mL. The cell suspension was then layered onto 3 mL of Histopaque 1083 (Sigma Chemical Co.) and centrifuged at 700 x g for 30 min. The macrophages were collected by removing the cells at the density gradient interface by aspiration with a Pasteur pipet. The cells were suspended in RPMI 1640 to a final volume of 13 mL and centrifuged at 450 x g for 10 min. The pellet was resuspended in 1-1.5 mL of RPMI 1640 and viable cells were counted by Trypan Blue dye exclusion. The isolated cells were visually identified as macrophages. Slides were prepared by centrifuging the 0.5 mL of purified sample onto glass slides using a Cytospin 2 centrifuge (Shandon Southern Products Ltd. Astmoor, U.K.). After the slides were left to dry overnight, the preparation was stained with STAT STAIN (VWR Scientific Inc., Brisbane, CA.). The samples were examined on a microscope using oil immersion (1000 X magnification).

Chemiluminescence Assay. A macrophage concentration of 2×10^6 cells/mL of RPMI 1640 was used in the assay. Four hundred μ L of cells plus 20 μ L of 0.002 M lucigenin (Sigma Chemical Co.) was pipetted into a cuvette and placed in an LKB 1251 luminometer (LKB, Stockholm, Sweden) set at 37°C. The luminometer controlled dispensers delivered 200 μ L of RPMI 1640, or activated zymosan to each sample at time zero of the assay. The total chemiluminescence (measured in mV) was recorded over a 15 sec period at 2-3 min intervals for each sample for up to 2 hr.

Adaptation of egg-propagated influenza A/PR/8 in mice. Influenza A/PR/8 virus was adapted in mice by four blind passages using egg-propagated virus (ATCC, Parklawn,

MD.) as the initial inoculum. For each passage, BALB/c mice, anaesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), were inoculated intranasally with 50 μ l of egg-propagated virus for the initial passage. At four days post infection, the mice were sacrificed and the lungs aseptically removed. The lungs were then homogenized in a tissue grinder with a mixture of 5 g sterile aluminium hydroxide powder and 10 mL phosphate buffered saline, pH 7.2 containing penicillin-G (100 μ g/mL), fungizone (0.25 μ g/mL) and streptomycin sulphate (100 μ g/mL). The ground lung extract was then centrifuged at 5000 X g for 15 min and the supernatant was used for re-inoculation into mice in subsequent passages. The supernatant from the fourth and final passage was inoculated into the allantoic cavity of embryonated hens' eggs and the eggs were incubated at 37°C for 4-5 days. The allantoic fluids were then isolated and pooled. The pooled allantoic fluid was assayed for virus infectivity by a mouse LD₅₀ assay.

LD₅₀ Determination for mouse-adapted influenza A virus. Pooled allantoic fluid from embryonated eggs infected with lung extract from the fourth passage in mice was diluted serially in sterile PBS. Balb/c mice, anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), were inoculated intranasally with 50 μ l of the virus dilutions (8 mice per group). At day 14 post infection, the number of mice which had survived the virus infection was recorded. The LD₅₀ value was calculated by the method of Reed and Muench [1].

Influenza protection. The volume for intranasal administration of LIP- γ IFN was 50 μ l. For intranasal administration, mice were anesthetized with sodium pentobarbital as described above. When the animals were unconscious, they were carefully supported by hands with their nose up, and the material to be administered was gently applied with a micro-pipettor into the nostrils. The applied volume was naturally inhaled into the lungs.

In the study of prophylactic treatment of mice against influenza A infection with interferon, groups of sodium pentobarbital anesthetized mice (10 mice per group) were inoculated intranasally with either 50 μ l γ IFN (1000 U per mouse), γ IFN encapsulated within liposomes (LIP- γ IFN 1 μ mol total lipid containing 1000 U γ IFN per mouse, or with sham liposomes (sham LIP, 1 μ mole total lipid per mouse, no γ IFN). The mice were

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pre-treated at 24 hour intervals beginning at either 1, 2, 3, or 4 days before intranasal challenge with 10 LD₅₀ of mouse-adapted influenza A/PR/8 virus. At day 14 post virus challenge, the number of mice surviving the virus challenge was recorded.

Statistics. Statistical comparisons of the mortality and survival rates among groups of mice were performed by one-way analysis of variance (ANOVA), and were calculated using the Multivariate General Linear Hypothesis (MGLH) module of Systat computer software program (Evanston, IL.).

RESULTS

Macrophage Isolation. The average yield of macrophages from both immunomodulator-treated and control mice is shown in Table I. Treatment with an immunomodulator (MDP or γ IFN) or with sham liposomes increased the macrophage yield over 3-fold. Mice receiving liposome encapsulated γ IFN gave the highest yield with nearly a 5-fold increase over the PBS control group.

Macrophage Stimulation. Activation of peritoneal macrophage phagocytic activity was measured by the chemiluminescence assay. Activation of phagocytic activity was observed with both MDP and γ IFN. Activation of peritoneal macrophages from MDP-treated mice was not detectable until after two days post treatment (Figure 1). Activation of macrophages isolated from γ IFN treated mice was evident at both 1 and 2 days post-treatment but after 3 days phagocytic activity decreased to near control levels (Figure 2). Activation of peritoneal macrophages from mice treated with liposome-encapsulated γ IFN was more pronounced than that of both mice treated with PBS or with free γ IFN. At 24 hours post-treatment LIP- γ IFN was shown to be more effective than γ IFN or LIP alone (Figure 3).

LD₅₀ Determination for mouse-adapted influenza A virus. The LD₅₀ determination in mice infected intranasally with mouse-adapted influenza A/PR/8 is shown in Table II. All mice infected with the mouse-adapted virus at a dilution of 10⁻⁵ or lower died from the infection. The LD₅₀ calculated by the method of Reed and Munch, was 10^{-5.21}. The

50% survival time, defined as the time by which 50% of the mice died from infection with 10 LD₅₀ of the virus, was found to be around 7 days.

Interferon-Mediated Influenza Protection. The efficacies of free and liposome-encapsulated γ IFN in protection against influenza A infection were compared in the following experiments. Groups of mice were pretreated with either PBS, sham LIP, free γ IFN or LIP- γ IFN on various days prior to intranasal infection with 10 LD₅₀ of influenza A virus. As summarized in Table III, groups of 10 mice pretreated with either PBS or sham LIP were completely unprotected against infection with the influenza virus. When treated with free or unencapsulated γ IFN there was a small but significant, 20% survival rate in the groups of mice only after 3-4 repeated doses at 24 hour intervals before viral inoculation. Mice pretreated with LIP- γ IFN had the greatest survival rate. A single dose of LIP- γ IFN was as effective as 3-4 doses of γ IFN (ie. 20% survival). Repeated intranasal doses of LIP- γ IFN 2 to 4 days prior to inoculation with influenza virus resulted in survival rates of 50 to 70 %. The LIP- γ INF had its highest efficacy when administered in three doses at 24 hour intervals prior to infection with influenza A resulting in a 70 % survival rate (Table III).

DISCUSSION

Interferons are unique hormone-like proteins which have non-specific antiviral and immunomodulatory activities [6,7]. The antiviral activity results from the ability of interferon to induce the synthesis of host proteins which inhibit viral replication. Interferon also enhances a number components of cellular immunity including, expression of histocompatibility antigens, natural killer cell activity of lymphocytes and antibody-dependent cell-mediated cytotoxicity [7]. These features make it an ideal candidate for prophylaxis against wide variety of infectious and BW agents. For example, three interferon types have recently been shown to protect mice from a wide range of virus species [15]. In addition, previous studies have suggested a role for free interferon for prophylaxis against respiratory disease [12] However, since this protein is a small protein it is subject to clearance and degradation from the body in a relatively short period of

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time [13]. The results of this study demonstrate how the efficacy of γ INF's antiviral and immunomodulatory activity could be enhanced by encapsulation into liposomes.

In the first instance we have demonstrated *in vitro* that immunomodulators can be used to elicit a general, non-specific immune response to the hosts in which they are introduced. Studies measuring enhanced phagocytic activity of peritoneal macrophages demonstrates that the effect is a general feature of different types of immunomodulator and not just a specific artifact of γ INF. It is interesting to note that there was a significant increase in the peritoneal macrophage yield upon administration of sham liposomes to the peritoneum. While subsequent *in vivo* data does not suggest this results in an increased protection against viral infection, this phenomenon may be important where conditions requiring increased macrophage numbers or activity is required. Where encapsulated γ INF was administered there was the largest increase in both macrophage number and phagocytic activity.

Secondly, the *in vivo* experiments have demonstrated that the efficacy of γ INF for protection against infection by influenza A virus is enhanced by liposome encapsulation. Protection against influenza mortality could be detected in mice pretreated with free and liposome encapsulated γ INF. However, LIP- γ INF after 1 day of pretreatment was as effective as 3-4 days of pretreatment of free γ INF. Three days of pretreatment with LIP- γ INF resulted in up to 70% survival of infected mice after 14 days. This remarkable increase in γ INF efficacy is likely to be the result of the increased retention of γ INF in the lungs afforded by liposome encapsulation. Indeed, radiotracer studies using ^{125}I -IgG indicated that lung retention of liposome-encapsulated IgG was 3-fold more than unencapsulated IgG even at 24 hr post administration [20]. The observed decrease in survival after 4 days of LIP- γ INF pretreatment may reflect a narrow optimum dose range for γ INF which has been observed when the non-encapsulated form was used in humans [10]

Administration of γ INF to the host in this manner significantly delays the onset of the morbidity and mortality associated with the viral disease. A major benefit of this form of treatment is that it allows the host immune system time to elicit a natural specific immune response to fight the infection. Such an advantage could be most significant in

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the protection of personnel exposed to infectious BW agents. However one of the major drawbacks of interferon therapy is that interferons are toxic molecules which can cause severe toxicities to the central nervous system, renal and cardiac functions [7, 19]. Liposome encapsulation may prove extremely valuable in interferon immunotherapy because liposomes could dramatically reduce interferon toxicities through their slow release characteristics. Valuable information may be obtained from future studies were liposome encapsulated γ IFN may be administered both prior to and during the course of viral infections caused by influenza or possibly other potential BW agents of viral nature. Additional studies are also required pertaining to various mechanisms to deliver the liposome encapsulated drugs in a form appropriate for human use.

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TABLE I

**Yield of peritoneal macrophages
following administration of free or
liposome-encapsulated immunomodulators**

Test Group	Cells Harvested per Mouse (10^6)
PBS Control	1.1 ± 0.6
MDP	3.7 ± 1.6
Free γ IFN	2.5 ± 1.4
Sham liposomes	3.7 ± 1.6
LIP- γ IFN	5.3 ± 2.0

\pm standard deviation of the mean

TABLE II

**LD₅₀ Determination in Mice Infected Intranasally
with Mouse-Adapted Influenza A Viruses**

Log of Virus Dilution	No. of Survivors at Day 14 post virus challenge	% Mortality
-4	0/8	100
-5	0/8	100
-6	5/8	37
-7	8/8	0

TABLE III

The effect of γ IFN and LIP- γ IFN pretreatment on the protection of mice against intranasal challenge with 10 LD₅₀ of mouse adapted influenza A virus.

Group	Days Before Infection	No. of Survivors at day 14 post infection	% Survival
PBS	3, 2, 1	0/10	0
Sham LIP	3, 2, 1	0/10	0
γ IFN	1	0/10	0
	2, 1	0/10	0
	3, 2, 1	2/10	20
	4, 3, 2, 1	2/10	20
LIP- γ IFN	1	2/10	20
	2, 1	5/10	50
	3, 2, 1	7/10	70
	4, 3, 2, 1	6/10	60

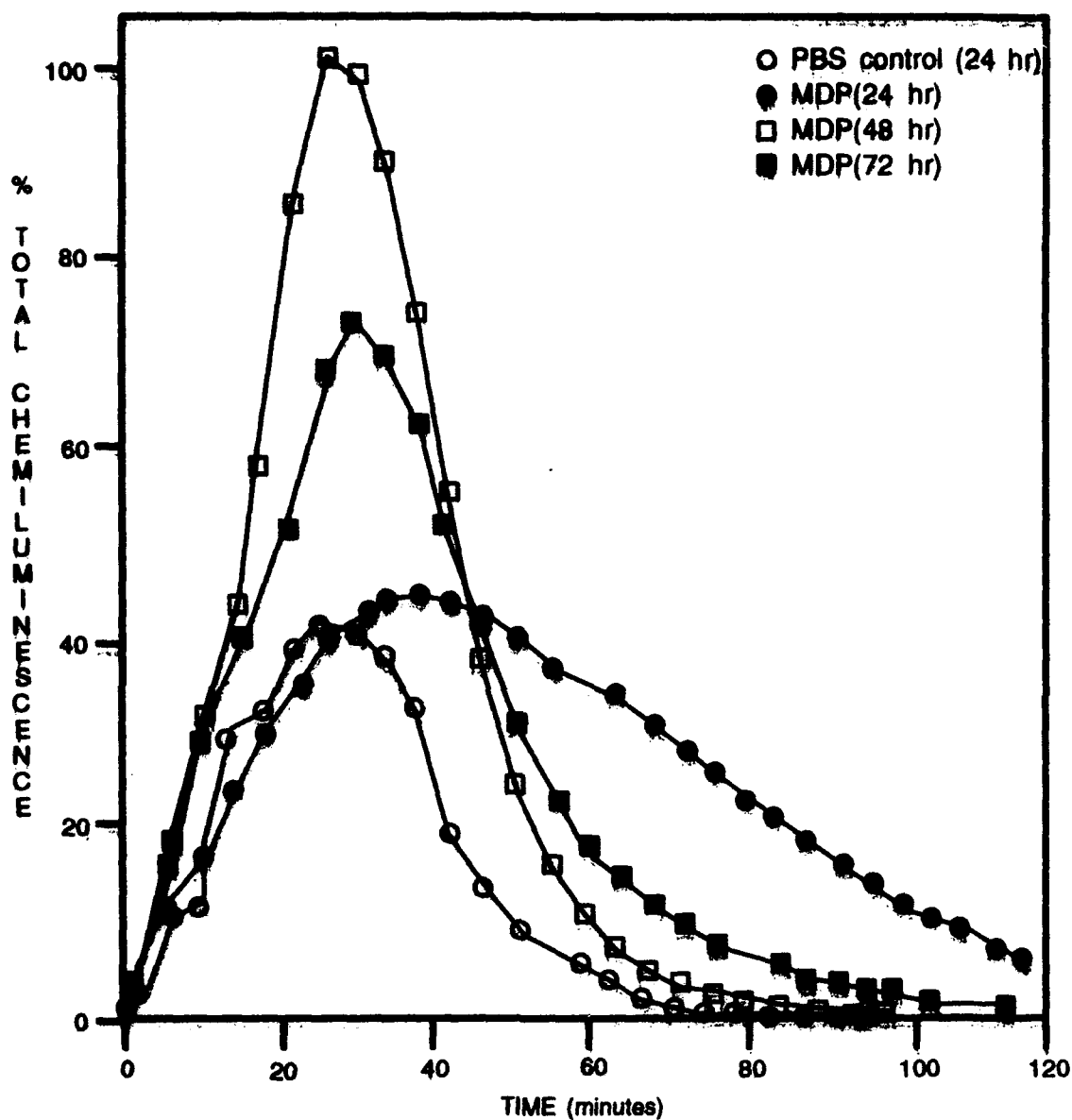
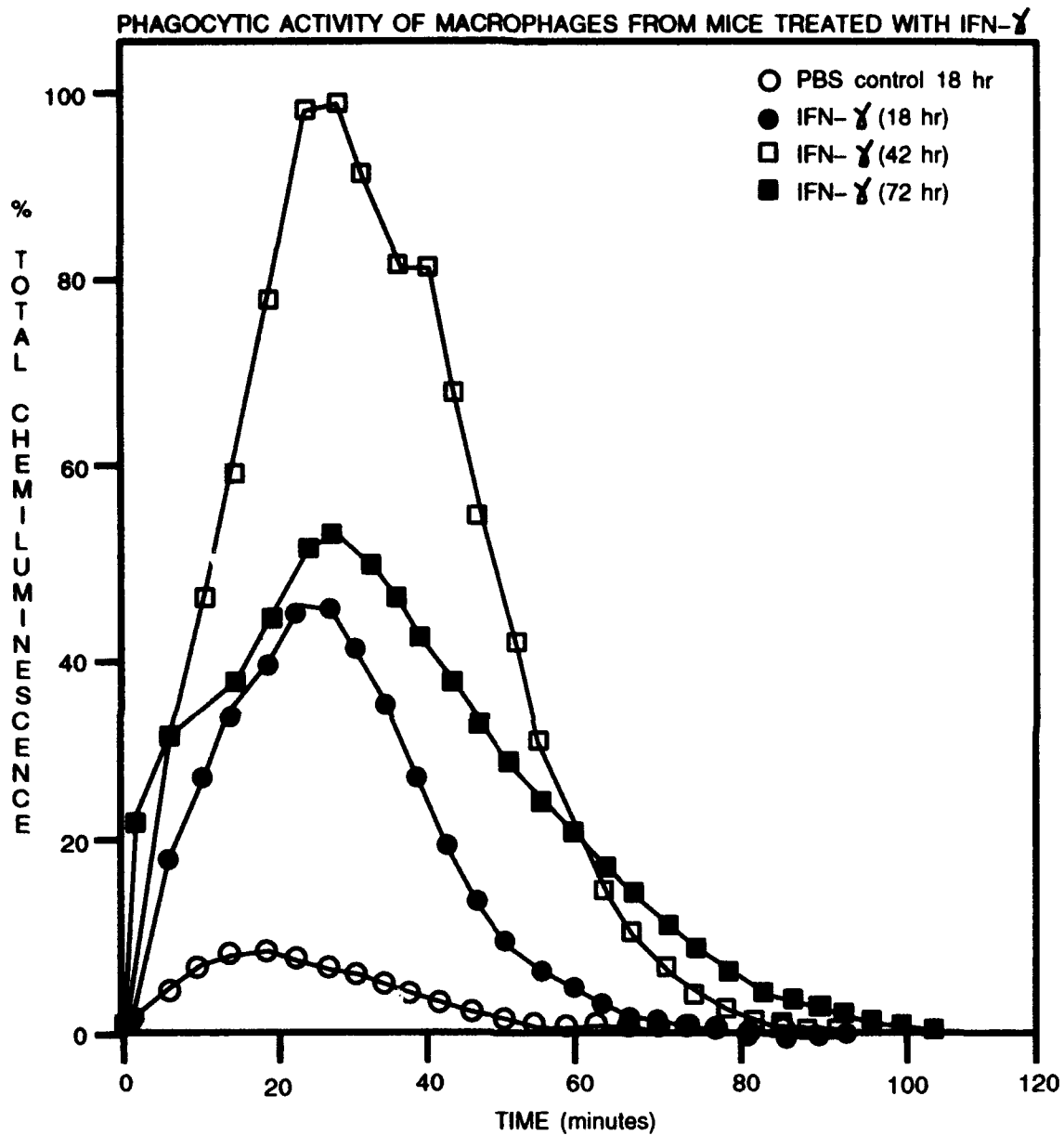


Figure 1

Chemiluminescence assay of mice treated with MDP. Treatment with free MDP. The cell concentrations used in the assay are 4.06×10^6 cells/ml (PBS control, 24 hr), 4.08×10^6 cells/ml (MDP, 24 hr), 4.11×10^6 cells/ml (MDP, 48 hr) and 3.86×10^6 cells/ml (MDP, 72 hr).

**Figure 2**

Chemiluminescence assay of mice treated with free IFN- γ . The cell concentrations used in the assay were 2.15×10^6 cells/ml (PBS control, 18 hr), 2.3×10^6 cells/ml (IFN- γ , 18hr), 2.67×10^6 cells/ml (IFN- γ , 42 hr) and 2.73×10^6 cells/ml (IFN- γ , 72 hr).

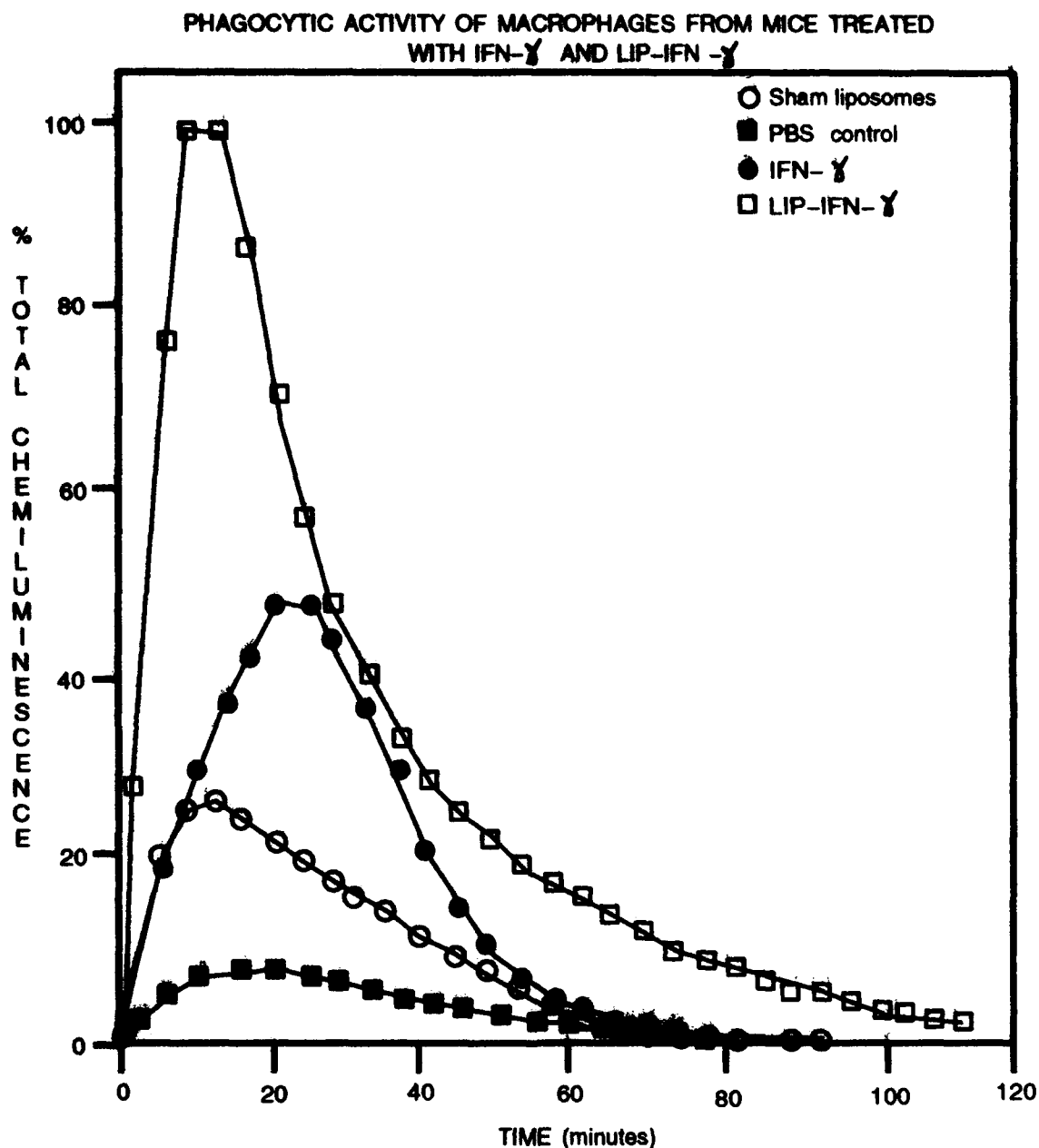


Figure 3

Chemiluminescence assay of mice treated with IFN- γ and LIP-IFN- γ . The cell concentrations used in the assay for the sham-LIP, IFN- γ , and LIP-IFN- γ were 2.3×10^6 cells/ml, in each assay. Macrophage phagocytic activity was assayed 18 hr post treatment. Phagocytic activity was assayed as described in materials and methods.

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The ability of liposome-encapsulated gamma interferon (LIP- γ IFN) to stimulate mouse cell-mediated immunity was assessed both in vivo and in vitro. The enhancement of the cell-mediated immune response was demonstrated in vitro by a chemiluminescent assay which measured the phagocytic activity of peritoneal macrophages. Peritoneal macrophages harvested from mice treated with gamma interferon (γ IFN) or muramyldipeptide showed significant increases in both macrophage yield as well as in ability to phagocytize zymosan particles. However, when treated with γ IFN encapsulated within liposomes both macrophages yield and phagocytic activity further increased by at least 100% over unencapsulated γ IFN. Using the in vivo influenza mouse protection model, intranasally administered LIP- γ IFN resulted in a 70% survival rate to mice challenged intranasally with 10 LD₅₀ doses of influenza A/PR/8 virus compared with a 20% survival rate with free γ IFN. Together these results suggest that liposome encapsulation increases γ IFN efficacy in providing non-specific stimulation of the cell-mediated immune system.

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